35. (amended) A substantially pure form of human α PDGFR protein according to claim 34, wherein the receptor protein contains a signal peptide and has the amino acid sequence of amino acids 1-1089 as shown in Figure 3.

36. (amended) A substantially pure form of human α PDGFR protein according to claim 34, wherein the receptor protein has the amino acid sequence of amino acids 24-1089 as shown in Figure 3.

REMARKS

Claims 4-6, 22 and 25-36 are pending in this application. Claims 4, 5, 25, 32 and 34-36 have been amended for clarity to more particularly define the invention. The title of the invention has also been amended herein for clarity. It is believed that no new matter has been added by these amendments. In light of these amendments and the following remarks, applicants respectfully request reconsideration of this application and allowance of the pending claims to issue.

Applicants acknowledge that claims 22 and 26-33 are allowed.

Applicants wish to draw the Examiner's attention to the Substitute Power of Attorney and Associate Power of Attorney included herewith. These documents were faxed to the Examiner on December 29, 1997. However, the February 14, 1998 Notification of Change due to PTO Move and Consolidation was mailed to the attorneys who were previously handling this application. Applicants request that these Power of Attorney documents be entered into the present application and that all future correspondence regarding this application be directed as indicated therein.

I. Title of the Invention

The Office Action states that the title of the invention is not descriptive and that a new title is required which is clearly indicative of the invention to which the claims are directed.

The title of the present invention has been amended herein as follows: COMPOSITIONS OF ALPHA PLATELET-DERIVED GROWTH RECEPTOR NUCLEIC ACID AND PROTEIN AND METHOD OF MAKING. Thus, applicants believe that the new title is clearly indicative of the invention to which the claims are directed and respectfully request entry of the new title and withdrawal of this objection.

II. Rejection under 35 U.S. § 112, second paragraph

Claims 4-6 and 25 are rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. Specifically, the Office Action states that claims 4-6 and 25 depend either directly or indirectly from claim 23 which is a canceled claim and are therefore unclear due to their dependence. The Office Action further states that although applicants have argued that claim 25 depends from claim 26, such claim 26 dependence has not been amended into claim 25.

Claim 25 has been amended herein to depend directly from claim 26, thereby providing proper dependency of claims 4-6 and 25. Thus, this rejection has been mooted and applicants respectfully request its withdrawal and allowance of the pending claims to issue.

III. Rejection under 35 U.S.C. § 102(b)/103(a)

Claims 34-36 are rejected under 35 U.S.C. § 102(b) as allegedly anticipated by, or in the alternative, under 35 U.S.C. § 103(a) as allegedly obvious over either Hart et al. or Betsholtz et al., taken in view of Raines et al. and further in view of Hart (U.S. Patent No. 5,094,941.

Specifically, the Office Action states that this rejection is necessitated by amendment which effectively replaced claim 7 with presently pending claims 34-36. The Office Action further states that the basis for this rejection is summarized in the 2/25/97 Office Action and herein applied to claims 34-36 because applicants still have the burden of appropriately showing distinctness of the present claims over the reference disclosures.

Specifically, in the 2/25/97 Office Action, it is stated that the PDGF receptors isolated by Hart et al. are disclosed to include an approximately 130 kDa form and also shown as measured as having a molecular weight of 128 kDa on Western blot. The 2/25/97 Office Action further states that Betsholtz et al. disclose a 115 kDa protein as a protein expressed as a result of PDGF stimulation of human fibroblast cells and that therefore, the receptor of either Hart et al. or Betsholtz et al. meets the basic characteristics as instantly disclosed but is not characterized further such as regarding which PDGF isoforms are specifically bound by the receptor as is instantly studied and defined for the alpha form of the human PDGF receptor protein. The 2/25/97 Office Action goes on to state that in the instant application, the receptor has the same molecular weight as the prior art receptor and binds PDGF as a PDGF receptor must and that several forms of the PDGF receptor are disclosed in Hart et al., which allegedly indicates that both the alpha and beta form at least are therein disclosed. The 2/25/97 Office Action further notes that the instant disclosure further characterizes the alpha receptor regarding PDGF isoform binding as well as amino acid sequence, which further measurements appear to be merely the measurement of inherent properties of the prior art protein which meets the basic characterization also.

The 2/25/97 Office Action also states that although Hart et al. and Betsholtz et al. disclose the basic characteristics of the alpha PDGF receptor including binding PDGF, no detailed characterization of PDGF is discussed. The 2/25/97 Office Action then points out that Raines et al. reveals that multiple forms of PDGF are contained in the PDGF preparation used in

these publications and that Hart et al. Patent No. 5,094,941 discloses this PDGF as containing AA , AB and BB isoforms, thus including all forms that may be bound by the receptor of Hart et al., although not specifically characterized therein. On this basis, the Examiner alleges that it would have been obvious to someone of ordinary skill in the art at the time this invention was made to isolate PDGF receptor as given in either Hart et al. or Betsholtz et al. and characterize it to determine the inherent properties as instantly disclosed because the different isoforms of PDGF were known in Hart et al., Patent No. 5,094,941, which gives the suggestion that a form of PDGF receptor binds all three isoforms. The 2/25/97 Office Action further contends that sequencing per se of a protein is also the determination of an inherent property of a protein, even though performed by genetic engineering methods.

Claim 34 has been amended herein to recite a substantially pure form of human type α platelet derived growth factor receptor (α PDGFR) protein having a species with a molecular weight of 180-185 kDa and a species with a molecular weight of 160 kDa as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and having the amino acid sequence selected from the group consisting of (a) amino acids 1-1089 of Figure 3; and (b) amino acids 24-1089 of Figure 3.

Claims 35 and 36 recite a substantially pure form of human α PDGFR protein according to claim 34, wherein the receptor protein contains a signal peptide and has the amino acid sequence 1-1089 as shown in Figure 3 or wherein the signal peptide has been cleaved and the receptor protein has the amino acid sequence 24-1089 as shown in Figure 3, respectively.

Support for the amendment to claim 34 can be found throughout the specification, in which it is described that the molecular weight of each of the two species of the αPDGR protein of this invention was determined to be 180-185 kDa and 160 kDa, respectively, by Western blot and immunoprecipitation analyses. In particular, on page 31 lines 27 through page 32, line 3 of

the specification, it is stated: "Proteins (100 μg per lane) were resolved by electrophoresis in 7% SDS-polyacrylamide gels, transferred to nitrocellulose filters and probed by immunoblot analysis (with or without peptide blocking) using ^{125}I-Protein A (51)." Furthermore, it is stated in the specification on page 32, line 24 through page 33, line 1: "...cell lysates were immunoprecipitated with anti-peptide antisera. Total cell lysates or immunoprecipitates were analyzed by immunoblotting with antibodies to the receptors or to phosphotyrosine (anti-P-Tyr) (54)."

The receptor antibodies used in these immunoprecipitations and immunoblot analyses are described in the specification on page 31, lines 3-9 and on page 43, line 27 through page 44, line 3, the latter of which states: "Antibodies specific for either the novel or known PDGF receptor protein. In an effort to identify the protein product of the new gene, antisera to peptides were prepared based on its predicted sequence. Analogous regions of the predicted sequence of the known PDGF-R were utilized to generate antisera as well."

The results of these immunoprecipitation and immunoblot experiments are described in the specification on page 44, lines 13-25 as follows: "Western blot analysis of M426 cells with antisera (anti-T11) directed against the T11 gene product revealed 180 kd and 160 kd protein species which were specifically competed by the immunizing peptide. The anti-PDGF-R peptide serum (designated anti-HPR) detected 180 kd and 165 kd proteins in the same cells. Western blot analysis of 8387 cells revealed 180 and 165 kd species, which were recognized by the anti-HPR, but not by anti-T11 serum. Conversely, A204 cells contained 180 and 160 kd species which were specifically detected by anti-T11, but not recognized by anti-HPR serum." These data are depicted in Figure 7A.

The specification additionally states, on page 45, lines 11-20: "Transient expression in COS-1 cells (the transfection of COS-1 cells with vectors carrying T11 cDNA or PDGF-R cDNA

is described in the specification at page 32, lines 5-7 and in the legend of Figure 7) led to the specific detection of the T11 gene products as 185 kd and 160 kd species (Fig. 7B) whereas the PDGF-R appeared as 185 kd and 165 kd proteins. The respective lower MW forms of each receptor did not vary in size among the cells analyzed. However, some different sizes of the higher MW species were observed, which were likely due to cell specific differences in glycosylation."

Furthermore, the αPDGFR protein of this invention is described on page 47, lines 12-27 and in Figure 9 as having a 180 kd protein species as determined by immunoprecipitation followed by immunoblotting with the antisera of this invention.

Thus, the specification clearly describes that the αPDGFR protein of this invention is characterized as having a species with a molecular weight of 180-185 kDa and a species with a molecular weight of 160 kDa, as determined by SDS-polyacrylamide gel electrophoresis, in immunoprecipitation and immunoblot experiments. In addition, the αPDGFR protein of the present invention can have the amino acid sequence as shown in Figure 3. Thus, claim 34 as amended herein is fully supported by the teachings of the specification.

In contrast, Hart et al. disclose PDGF receptors, produced in human dermal fibroblasts, having a molecular weight of 180, 164 and 130 kDa, respectively, as determined by immunoblot and immunoprecipitation analyses using a monoclonal antibody designated PR7212 which was described in the Hart et al. reference as specifically recognizing "PDGFR" and which was subsequently identified to be a monoclonal antibody specific for βPDGFR. In particular, applicants provide herewith as Exhibit A, a copy of a publication entitled "Two different subunits associate to create isoform-specific platelet-derived growth factor receptors." (Seifert et al., J. Biol. Chem. 264(15):8771-8778 (1989)), which includes as co-authors all of the co-authors of the Hart et al. reference of this rejection. Seifert et al. describe experiments which were

carried out to determine that monoclonal antibody PR7212 recognizes only βPDGFR and does not bind αPDGFR. In particular, it is stated on page 8774, second column, last full paragraph of the Seifert et al. reference: "Monoclonal Antibody PR7212 Recognizes the β-Subunit of the PDGF Receptor- We have recently generated a monoclonal antibody, PR7212, against the PDGF receptor (Hart et al., 1987). Two experiments designed to determined whether the antibody recognizes the α- or β-subunit of the PDGF receptor demonstrated that it recognizes only the β-subunit." Seifert et al. then go on to describe these two experiments, starting on the bottom of the second column of page 8774 and continuing through the first full paragraph of page 8775. Because all of the PDGF receptors of the Hart et al. reference were characterized by either immunoprecipitation or immunoblot with monoclonal antibody PR7212, all of the PDGF receptors of the Hart et al. reference are βPDGF receptors. Thus, Hart et al. clearly provide no teaching or suggestion of the αPDGF receptor of this invention and accordingly, the αPDGF receptor of this invention is neither anticipated nor rendered obvious by this reference.

Furthermore, Betsholtz et al. discloses a PDGF receptor having a molecular weight of 115 kDa as determined by SDS-PAGE, which is clearly not the αPDGFR of the present invention, which has been shown to have a species with a molecular weight of 180-185 kDa and a species with a molecular weight of 160 kDa as determined by SDS-PAGE. Thus, the Betsholtz et al. paper does not teach or suggest the αPDGFR protein of this invention. Accordingly, the αPDGFR of the present invention is neither anticipated nor rendered obvious by Betsholtz et al.

In addition, the teachings of Raines et al. and Hart et al. U.S. Patent No. 5,094,941 have no relevance in this rejection because applicants have provided ample evidence that none of the PDGF receptors of either Hart et al. or Betsholtz et al. are the αPDGF of the present invention, rendering moot any discussion of what isoforms of PDGF were present in the PDGF preparations used in the experiments described in Hart et al. and Betsholtz et al. For these reasons, applicants

believe that this rejection has been overcome and respectfully request its withdrawal and allowance of the pending claims to issue.

IV. Additional claim amendments

Applicants also wish to point out that claims 4, 5 and 25 have been amended herein to recite a cDNA rather than a DNA or DNA segment, for consistency with the language of the claims from which these claims are dependent. In addition, allowed claim 32 has been amended to include the phrase "nucleotide sequence encoding" for clarity and consistency with the language of claim 31. Finally, claims 35 and 36 have been amended to include the phrase "of amino acids" for clarity and consistency with the language of claim 34. Support for these clarifying amendments can be found throughout the specification as well as in the language of the pending and allowed claims. Applicants assert that no new matter has been added by these amendments and respectfully request their entry.

Pursuant to the above amendments and remarks, reconsideration and allowance of the pending application is believed to be warranted. The Examiner is invited and encouraged to directly contact the undersigned if such contact may enhance the efficient prosecution of this application to issue.

A check in the amount of $1260.00 ($950.00 for the three month extension fee and $310.00 for the Notice of Appeal fee), a Request for Extension of Time and a Notice of Appeal are enclosed. Applicants believe this amount to be correct, however, the Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to

Deposit Account No. 14-0629.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: BOX AF, Assistant Commissioner of Patents, Washington, D.C. 20231, on June 8, 1998.

Mary L. Miller — June 8, 1998
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Two Different Subunits Associate to Create Isoform-specific Platelet-derived Growth Factor Receptors*

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Recent evidence has demonstrated that there is more than one form of platelet-derived growth factor (PDGF) receptor and that these receptors differ in their specificity for the multiple isoforms of PDGF. We present evidence that high affinity binding of PDGF requires association of two different receptor subunits: an α-subunit that can bind either a B- or an A-chain of PDGF, and a β-subunit that can bind only a B-chain. The α- and β-subunits appear to be similar in size but can be distinguished by binding specificity and by an antireceptor monoclonal antibody, PR7212, which recognizes only the β-subunit. In the absence of PDGF, these subunits either exist separately or form rapidly reversible complexes. In the presence of PDGF, receptor subunits of appropriate specificity interact with a PDGF molecule to form a high affinity complex. Both the absolute and relative numbers of these two PDGF receptor subunits vary on different cell types and correspond to differences in the mitogenic sensitivity of cells to the different PDGF isoforms.

Binding of PDGF[1] to its cell surface receptor initiates many biological responses in cells of mesenchymal origin, including increased metabolic rates, chemotaxis, and proliferation (reviewed in Ross *et al.*, 1986). Cells that respond to PDGF have been found to possess high affinity binding sites for ^{125}I-PDGF (reviewed in Bowen-Pope *et al.*, 1985). Studies in which the receptor was identified by affinity cross-linking to ^{125}I-PDGF or by PDGF-stimulated incorporation of ^{32}P into a specific membrane protein suggested a molecular mass for the receptor of about 170–180 kDa (Ek *et al.*, 1982; Glenn *et al.*, 1982; Heldin *et al.*, 1983; Frackelton *et al.*, 1984; Williams *et al.*, 1984). This receptor molecule is composed of a 120-kDa protein core (Yarden *et al.*, 1986) which is rapidly converted to a 160-kDa glycosylated precursor and subsequently matures to a 180-kDa mature cell surface form (Claesson-Welsh *et al.*, 1987; Hart *et al.*, 1987; Keating and Williams, 1987). A mouse PDGF receptor protein has been purified and partially sequenced (Yarden *et al.*, 1986), and both mouse and human PDGF receptor cDNAs have been cloned, sequenced, and expressed (Yarden *et al.*, 1986; Claesson-Welsh *et al.*, 1988; Escobedo *et al.*, 1988; Gronwald *et al.*, 1988).

PDGF itself is composed of two closely related but nonidentical protein subunits derived from different genes located on separate chromosomes (Dalla Favera *et al.*, 1982; Swan *et al.*, 1982; Betsholtz *et al.*, 1986). Each of the two homodimeric forms of PDGF, PDGF-AA and PDGF-BB, has been purified from natural sources and found to be functionally active, as is the heterodimeric molecule, PDGF-AB (Stroobant and Waterfield, 1984; Hart *et al.*, 1988; Nister *et al.*, 1988). Cloning of the PDGF genes has resulted in the availability of purified recombinant PDGF homodimers from the genes expressed in yeast (Kelly *et al.*, 1985; Heldin *et al.*, 1988; Hart *et al.*, 1988; Escobedo *et al.*, 1988), and highly purified PDGF heterodimers have been purified from human platelets by established techniques followed by affinity chromatography using PDGF chain-specific monoclonal antibodies to separate the heterodimers from both homodimers (Hart *et al.*, 1988).

Competition binding studies have recently shown that different isoforms of PDGF bind to different classes of PDGF receptor (Hart *et al.*, 1988; Heldin *et al.*, 1988; Nister *et al.*, 1988). In our initial publications (Hart *et al.*, 1988; Gronwald *et al.*, 1988), we proposed that the PDGF-binding properties of diploid human fibroblasts could be explained by postulating the existence of two classes of PDGF receptor which were presumed to consist of single transmembrane proteins: a "B-receptor" that binds only PDGF-BB, and an "A/B-receptor" that binds all three isoforms, *i.e.* a "monomeric receptor model." The studies presented here indicate that this model was incomplete. We now propose that the PDGF receptor consists of combinations of two different subunits (designated "α" and "β") which dimerize to form three distinct PDGF receptors that differ in their ability to bind the three PDGF isoforms. The α-subunit binds either the A- or B-chain of the PDGF molecule; the β-subunit binds only the B-chain of PDGF. Thus, αα-receptors bind all three PDGF isoforms, αβ-receptors bind PDGF-AB and PDGF-BB, and ββ-receptors bind only PDGF-BB. We will call this the "receptor subunit model." The total and relative numbers of these PDGF receptor subunits vary on different cell types and apparently determine the sensitivity of these cell types to the different isoforms of PDGF.

EXPERIMENTAL PROCEDURES

Cell Lines—Diploid human dermal fibroblasts (SK5) were isolated by explant from a normal adult donor and used at passage 7–10. Foreskin fibroblasts, AG1523, cultured from a newborn male were obtained from The Human Genetic Mutant Cell Repository, Camden,

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[1] The abbreviations used are: PDGF, platelet-derived growth factor; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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NJ. Two human osteogenic sarcoma cell lines, U-2 OS and MG-63, were obtained from the American Type Culture Collection, Rockville, MD. Swiss/3T3 clone NR6 is an epidermal growth factor receptor-negative cell line obtained from H. Herschman, University of California, Los Angeles. Other Swiss/3T3 cell lines were examined and found to have similar PDGF-binding properties and mitogenic responsiveness as reported here for clone NR6. These cell lines were cultured in Dulbecco-Vogt-modified Eagle's minimal essential medium (GIBCO) supplemented with 5 or 10% calf serum or 10% fetal bovine serum (AG1523).

Ligands and Antibodies—The antireceptor monoclonal antibody PR7212 (Hart *et al.*, 1987), was purified from mouse ascites using protein A-Sepharose affinity chromatography.

Highly purified PDGF-AB, free of contaminating PDGF-AA or PDGF-BB, was obtained in the following manner. PDGF was purified from outdated human platelets through the CM-Sephadex step described by Raines and Ross (1982) and then further purified to remove the two PDGF homodimeric forms by monoclonal antibody affinity chromatography. The partially purified PDGF was passaged over Sephadex-bound monoclonal antibody 121.6.1.1.1 that binds the PDGF B-chain. The resulting PDGF preparation, which contains only PDGF-AB and PDGF-BB, was then passed over a second affinity column, Sephadex-bound monoclonal antibody 120.1.2.1.1 that binds PDGF-BB only. The flow-through, containing only PDGF-AB, was finally purified by reverse phase HPLC on a C-18 resin column and determined to be at least 95% pure by SDS-PAGE and amino acid analysis. This preparation is also 99.5% free of PDGF-BB as determined by ligand-specific binding assays (Bowen-Pope *et al.*, 1989).

PDGF-BB (109 amino acids) and the "endothelial form" (Collins *et al.*, 1987; Tong *et al.*, 1987) of PDGF-AA (110 amino acids) were recombinant molecules expressed in yeast using a system previously described by Kelly *et al.* (1985) to express v-*sis*. To perform binding studies of ^{125}I-PDGF-BB, a mutant version of PDGF-BB, in which the phenylalanine at position 23 was replaced by tyrosine, was developed and expressed in yeast. A partial characterization of this mutant form of PDGF will be described (Bowen-Pope *et al.*, 1989). These recombinant molecules were purified from yeast culture medium by cation exchange chromatography followed by reverse phase HPLC and were shown to be at least 95% pure by SDS-PAGE and amino acid analysis. These ligands have been used previously to demonstrate multiple classes of PDGF receptors (Hart *et al.*, 1988) and to determine the PDGF isoform composition of biological fluids (Bowen-Pope *et al.*, 1989).

PDGF-AB was radioiodinated by the iodine monochloride method (Bowen-Pope and Ross, 1982) to a specific activity of 3.3×10^4 cpm/ng. PR7212, PDGF-AA, and the tyrosine-containing mutant of PDGF-BB were all labeled using IODO-BEADS (Pierce Chemical Co.) to specific activities of 8.6×10^4, 4.6×10^4, and 4.0×10^5 cpm/ng, respectively.

Ligand Binding and Competition Assays—Binding assays were done according to the methods of Bowen-Pope and Ross (1982). Cells were plated in 24-well trays in serum-supplemented Dulbecco-Vogt-modified Eagle's minimal essential medium and grown to confluence. For saturation binding experiments, the cells were rinsed with cold binding rise (phosphate-buffered saline containing 1 mM $CaCl_2$ and 1 mg/ml BSA) and then incubated at 4 °C for 3 h with constant shaking in 1 ml/well of binding medium (Ham's F-12 medium buffered to pH 7.4 with 25 mM Hepes and supplemented with 0.25% BSA) containing increasing concentrations of ^{125}I-PDGF-AB or ^{125}I-PDGF-BB. Nonspecific binding was determined using 500 ng/ml of unlabeled PDGF-BB. The trays of cells were then washed three times with cold binding rinse, solubilized in 1 ml of solubilization buffer (1% Triton X-100, 1 mg/ml BSA), and counted on a Beckman Gamma-5500.

For competition binding of ^{125}I-PDGF, the cells were rinsed once with cold binding rinse and then incubated in 1 ml of binding medium containing increasing amounts of the indicated unlabeled PDGF isoform at 4 °C for 3 h with constant shaking. We have shown previously that PDGF binding reaches equilibrium conditions by 1 h at saturating concentrations of ^{125}I-PDGF (Bowen-Pope and Ross, 1982). Preliminary experiments showed that binding of PDGF-AA and PDGF-BB also reached equilibrium by 1–3 h and that the bound PDGF isoforms did not detectably dissociate during a 4-h 4 °C incubation in PDGF-free binding medium (data not shown). To determine whether the different PDGF isoforms bind to receptors recognized by ^{125}I-PR7212, it was necessary to incubate cells with PDGF at 37 °C, since PDGF does not directly compete for binding of PR7212 to PDGF receptors (Hart *et al.*, 1988). At this temperature, occupied PDGF receptors are down-regulated due to rapid internalization and degradation (Heldin *et al.*, 1982; Bowen-Pope and Ross, 1982). The medium was then replaced with binding medium containing the different ^{125}I-PDGF-labeled isoforms or ^{125}I-PR7212 at concentrations that were approximately one-fourth of their respective apparent K_d and incubated an additional 2 h at 4 °C with constant shaking. Nonspecific binding was determined for ^{125}I-PDGF binding using 500 ng/ml unlabeled PDGF-BB and for ^{125}I-PR7212 binding by both prior and simultaneous incubation with 20 μg/ml PR7212 and was subtracted before calculating the percent of control binding. The trays of cells were then washed three times with cold binding rinse, solubilized in 1 ml of solubilization buffer (1% Triton X-100, 1 mg/ml BSA), and counted on a Beckman Gamma-5500.

Immunoprecipitation of ^{125}I-PDGF-Receptor Complexes—Confluent 150-cm^2 dishes of SK5 cells were incubated for 2 h at 4 °C in 20 ml of binding medium containing the indicated ^{125}I-PDGF isoform. The cell monolayers were rinsed three times in cold binding rinse, scraped in phosphate-buffered saline, and pelleted at 2,000 × *g* for 5 min. The cell pellet was resuspended in extraction buffer (10% glycerol, 10 mM Hepes, 0.2% Triton X-100, pH 7.4) and immediately centrifuged at 10,000 × *g* for 1 min to pellet detergent-insoluble material. The supernatant was harvested and used for immunoprecipitation and soluble receptor assays.

For immunoprecipitation assays, duplicate 20-μl aliquots of cell extracts were incubated for 1 h at 21 °C with 50 μl of PR7212 ascites (20 μg/ml antibody concentration) or control mouse serum. Fifty μl of a 0.1-mg/ml rabbit anti-mouse IgG antibody was added and the incubation continued for 1 h. 500 μl of extraction buffer was added followed by 25 μl of fixed *Staphylococcus aureus* (Pansorbin). After vortexing and incubating for 30 min, the mixture was centrifuged for 1 min at 10,000 × *g*. The supernatants were aspirated and the pellets counted for radioactivity. Nonspecifically immunoprecipitated radioactivity, defined as the cpm pelleted in samples containing preimmune mouse IgG, ranged from 10 to 21% of total added cpm in the cell extract and was subtracted from the total immunoprecipitated cpm.

Immunoprecipitation of Metabolically Labeled 3T3 PDGF Receptors—Confluent monolayers of mouse 3T3 cells cultured in 150-mm dishes were incubated for 18 h at 37 °C with 2 mCi of [^{35}S]methionine (Tran^{35}S-label, ICN, Irvine, CA) in 10 ml of methionine-free minimal essential medium. The cells were washed with phosphate-buffered saline, lysed by the addition of 4 ml of TNEN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 0.2% BSA, and the lysates were clarified by centrifugation at 2,000 × *g* for 10 min. The lysates were preadsorbed by the addition of 100 μl of *S. aureus* (Pansorbin). The samples were incubated 15 min on ice, the *S. aureus* pelleted, and the supernatants harvested and used for immunoprecipitation.

One ml of [^{35}S]methionine-labeled 3T3 lysate (2.5×10^6 cell equivalents) was added to 0.5 ml of human dermal fibroblast (SK5) lysate (1×10^6 cell equivalents) and the mixture incubated for 90 min on ice in the presence of PDGF-AA, PDGF-AB, PDGF-BB (100 ng in 10 mM acetic acid) or an equivalent volume of acetic acid. The samples were then split into two equal volumes and incubated 1 h with 5 μg of either PR7212 or a control antibody followed by addition of rabbit anti-mouse IgG (1-h incubation) and then 50 μl of *S. aureus* (30-min incubation) to precipitate the immune complexes. The samples were washed four times with 1 ml of TNEN containing 0.2% BSA, once with TNEN alone, boiled in sample buffer containing 5% β-mercaptoethanol, and then separated by SDS-PAGE on an 8% gel. The gel was fixed (40% methanol, 10% acetic acid), incubated with Enlightening (Du Pont-New England Nuclear) for 30 min, dried, and exposed for autoradiography at −80 °C using Kodak XAR-5 film.

RESULTS

Different Cell Types Express Different Numbers and Ratios of PDGF-AB- and PDGF-BB-binding Sites—The monomeric receptor model predicts that the number of the two proposed classes of PDGF receptors can be determined by using PDGF-AB and PDGF-BB. PDGF-BB would bind to all PDGF receptor classes, and PDGF-AB would bind only to the A/B-receptor. We surveyed a variety of cells for their PDGF receptor phenotype by performing saturation binding experiments with ^{125}I-PDGF-AB and ^{125}I-PDGF-BB. Human adult skin fibroblasts (SK5) and a human osteosarcoma cell line (U-2 OS) both have about 5-fold more ^{125}I-PDGF-BB- than

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^{125}I-PDGF-AB-binding sites even though the total number of receptors on these cells differs by over 10-fold (Fig. 1, *a* and *c*). Half-maximal binding for the two PDGF isoforms occurred at concentrations of 0.3–0.35 ng/ml (11.5 pM) for ^{125}I-PDGF-AB and 1.0–1.25 ng/ml (50 pM) for ^{125}I-PDGF-BB. In contrast, Swiss/3T3 cells (NR6) and a different human osteosarcoma cell line (MG-63) have nearly equal numbers of PDGF receptors for ^{125}I-PDGF-AB and ^{125}I-PDGF-BB (Fig. 1, *b* and *d*) yet show a 5-fold difference in the total number of PDGF receptors. Thus, both the total number of PDGF receptors and the ratio of these two binding specificities can vary widely and independently of one another between different cell types.

Binding Competition Studies Indicate That PDGF-AA and PDGF-AB Do Not Bind to the Same Set of Receptors—The monomeric receptor model proposed that PDGF-BB binds to all classes of PDGF receptors. This conclusion was based on the ability of unlabeled PDGF-BB to compete for binding of all ^{125}I-labeled isoforms of PDGF (Hart *et al.*, 1988). In the present study, we found that in all cell types, binding of all ^{125}I-PDGF isoforms was inhibited by PDGF-BB to an equal or greater extent than by either PDGF-AA or PDGF-AB (data not shown). This result confirms that PDGF-BB binds to all detectable forms of the PDGF receptor. Nonspecific binding of all of the ^{125}I-PDGF isoforms was therefore determined as binding which was not competed by very high concentrations of PDGF-BB (500 ng/ml).

The pattern of binding competition using human fibroblasts (SK5 and AG1523) is shown in Table I. PDGF-AA and PDGF-AB both competed for nearly all of the binding of both ^{125}I-PDGF-AA and ^{125}I-PDGF-AB but competed for only a small fraction of ^{125}I-PDGF-BB binding. This is the pattern of binding competition which led us to propose that human fibroblasts express an abundant B-receptor that binds only PDGF-BB and a less abundant A/B-receptor that binds all three isoforms (Hart *et al.*, 1988). However, since fibroblasts express relatively few ^{125}I-PDGF-AB-binding sites, the proposed property of this less abundant A/B-receptor, *i.e.* that it binds all three isoforms, was difficult to test quantitatively. The saturation binding data presented in Fig. 1 suggest that human MG-63 and 3T3 cells express nearly equal numbers of ^{125}I-PDGF-AB- and ^{125}I-PDGF-BB-binding sites, *i.e.* that the A/B-receptor should be abundant enough to contribute substantially to total ^{125}I-PDGF-BB binding. Thus, it should be possible to use this cell type to determine quantitatively to what extent PDGF-AA and PDGF-AB compete for ^{125}I-PDGF-BB binding. Fig. 2*a* shows that PDGF-AA and PDGF-

TABLE I

Binding competition of the PDGF isoforms

Binding competition experiments were performed as described under "Experimental Procedures." Results are expressed as percent inhibition compared with control binding where no competing ligand is present and are presented as the average ± (S.D.) of at least two and in most cases three to four separate determinations.

Cell type	^{125}I-PDGF-BB		^{125}I-PDGF-AB	^{125}I-PDGF-AA
	AA	AB	AA	AB
SK5	5.5 (3.6)	20.0 (8.7)	92.3 (2.1)	96 (3.5)
AG1523	4.2 (1.2)	20.5 (5.5)	84.3 (3.3)	98 (1.4)
U-2 OS	4.0 (5.6)	5.0 (5.0)	77.5 (12.5)	91 (1.4)
MG63	55.5 (19.1)	75.0 (12.5)	95.3 (2.4)	99 (0.7)
NR6	45.3 (13.0)	78.0 (9.7)	84.5 (5.5)	96 (2.8)

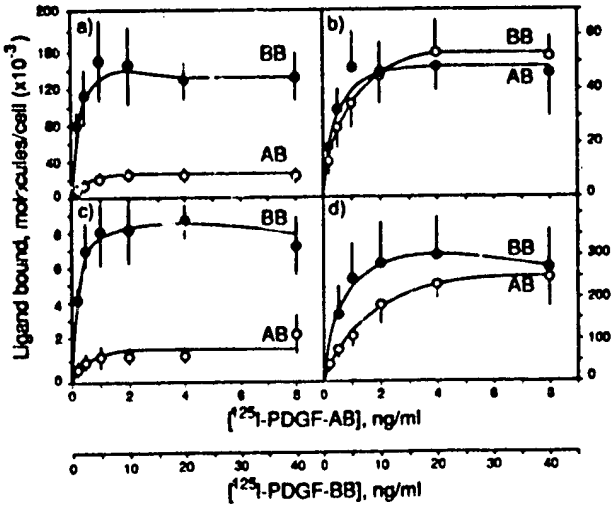

FIG. 1. **Number of PDGF-AB- and PDGF-BB-binding sites on different cell types.** Saturation binding analysis of ^{125}I-PDGF-AB and ^{125}I-PDGF-BB was performed as described under "Experimental Procedures." The results are plotted as the number of specifically bound ^{125}I-labeled ligand molecules/cell at each concentration tested and represent the average ± S.D. of three separate determinations. Nonspecific binding, determined at 2 ng/ml ^{125}I-PDGF-AB and 10 ng/ml ^{125}I-PDGF-BB, averaged 11.5% of the total bound counts for both ligands. Nonspecific binding to U-2 OS cells, which express very few PDGF receptors, was approximately 50% of the total bound counts. The cells examined were: *a*, SK5; *b*, MG-63; *c*, U-2 OS; and *d*, Swiss/3T3-NR6.

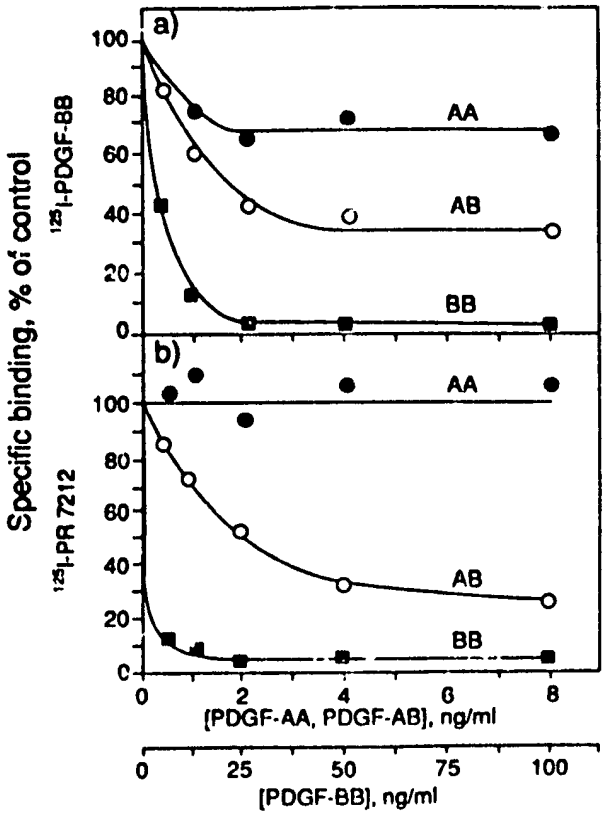

FIG. 2. **Competition for binding of the PDGF isoforms to MG-63 cells.** Binding competition/down-regulation by PDGF-AA, PDGF-AB, and PDGF-BB for (*a*) ^{125}I-PDGF-BB and (*b*) ^{125}I-PR7212 on MG-63 cells was determined as described under "Experimental Procedures." Results are expressed as the percent of specific control binding where no competing ligand was present. Total cpm bound/well was 3675 for ^{125}I-PDGF-BB and 825 for ^{125}I-PR7212. Nonspecific binding, expressed as the percentage of total bound counts, was 3% for ^{125}I-PDGF-BB and 9% for ^{125}I-PR7212.

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AB did in fact compete for ^{125}I-PDGF-BB binding. Half-maximal competition for ^{125}I-PDGF-BB binding occurred at a concentration of 0.7 ng/ml for PDGF-AA and PDGF-AB and a 3 ng/ml for PDGF-BB. However, PDGF-AA and PDGF-AB did not behave equivalently. PDGF-AB competed for two-thirds but PDGF-AA competed for only one-third of subsequent ^{125}I-PDGF-BB binding. Although the extent of competition by PDGF-AA and PDGF-AB for ^{125}I-PDGF-BB binding varied somewhat in different experiments, PDGF-AB always competed for more ^{125}I-PDGF-BB binding than did PDGF-AA, and usually the difference in binding competition by these two isoforms was about 2-fold (Table I).

This difference in the abilities of PDGF-AA and PDGF-AB to compete for ^{125}I-PDGF-BB binding cannot be explained by the previously proposed monomeric receptor model with only two receptor classes. The ability of PDGF-AB to compete for a larger fraction of ^{125}I-PDGF-BB than does PDGF-AA requires a third PDGF receptor class that binds PDGF-AB and PDGF-BB but not PDGF-AA (the "PDGF-X" receptor). However, the following observation cannot be accommodated by simply proposing a new class of monomeric receptor. The data in Table I would result in the calculation that PDGF A/B-receptors and X-receptors are expressed in nearly equal numbers on MG-63 osteosarcoma cells and 3T3 cells, *i.e.* PDGF-AB competed for approximately twice as much ^{125}I-PDGF-BB binding as did PDGF-AA. This, in turn, predicts that PDGF-AA should compete for only 50% of total ^{125}I-PDGF-AB binding. Instead, PDGF-AA consistently competed for nearly all binding by ^{125}I-PDGF-AB (Table I).

Subunit Model for the Structure of the PDGF Receptor—The receptor subunit model diagramed in Fig. 3 can readily accommodate all of the binding data. We propose that PDGF receptors require the dimeric association of two types of receptor subunits (α and β) which have the following binding specificities. The β-subunit can bind only a PDGF B-chain in a PDGF molecule, whereas the α-subunit can bind either a B-chain or an A-chain (Fig. 3*a*). These two subunits combine to form three different high affinity PDGF receptors: an $\alpha\alpha$-receptor, an $\alpha\beta$-receptor, and a $\beta\beta$-receptor (Fig. 3*b*). We propose dimerization rather than higher order multimerization, since dimerization is the simplest model that can account for the data and conforms to the presumptive dimeric nature of the PDGF molecule. (The possibility that higher order multimers form is being investigated.) The binding specificity of the PDGF receptor subunits predicts three receptor classes with the following binding specificities: $\alpha\alpha$-receptors bind all three PDGF isoforms; $\alpha\beta$-receptors bind only PDGF-AB and PDGF-BB; and $\beta\beta$-receptors bind only PDGF-BB (Fig. 3*c*). These three receptors would correspond to the previously proposed A/B-receptor, X-receptor, and B-receptor, respectively.

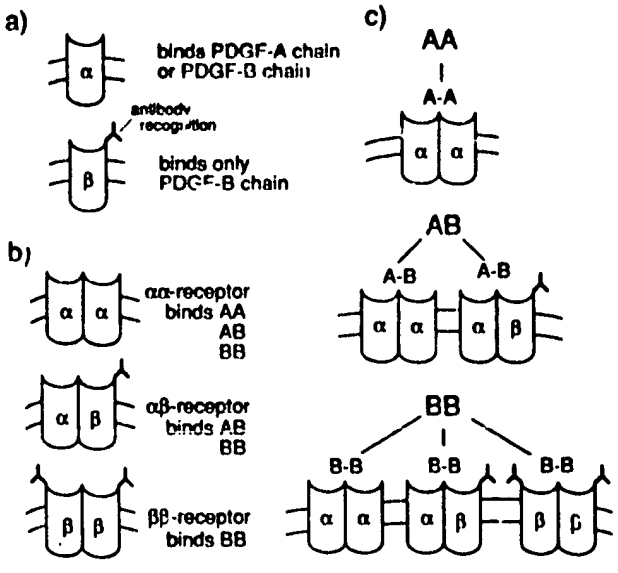

FIG. 3. **The PDGF receptor subunit model.** *a*, receptor subunits. Two types of PDGF receptor subunits exist: α-subunits that can bind PDGF A- and B-chains, and β-subunits that can bind only PDGF B-chains. Antibody PR7212 recognizes only the PDGF receptor β-subunit. Before ligand binding, PDGF receptor subunits are present on the cell surface as independent monomers or weakly associated dimers. *b*, receptor types. The two PDGF receptor subunits can form three types of high affinity PDGF receptors: $\alpha\alpha$-receptors, $\alpha\beta$-receptors, and $\beta\beta$-receptors. Antibody PR7212 recognizes the $\alpha\beta$- and $\beta\beta$-receptors via their β-subunits. *c*, receptor binding. PDGF-AA can only bind to PDGF $\alpha\alpha$-receptors, while PDGF-AB can bind to either PDGF $\alpha\alpha$- or $\alpha\beta$-receptors. PDGF-BB can bind to all three.

To understand the results of the competition binding experiments, it is important to realize that due to the binding specificities of the α- and β-subunits, PDGF-AA can bind only to $\alpha\alpha$-receptors, whereas PDGF-AB can bind to $\alpha\alpha$- and $\alpha\beta$-receptors, and PDGF-BB can bind to all three. Thus, the subunit model can explain the ability of PDGF-AA largely to eliminate ^{125}I-PDGF-AB binding since PDGF-AA would occupy all of the α-subunits that are required for PDGF-AB binding (Fig. 3*c*). The subunit model can also explain greater competition for ^{125}I-PDGF-BB binding by PDGF-AB than by PDGF-AA (Fig. 2*a* and Table I) because it predicts that PDGF-AB can bind to $\alpha\beta$-receptors and therefore recruit one β-subunit for each α-subunit it occupies, whereas PDGF-AA can only occupy α-subunits (Fig. 3*c*). Since both of these subunits contribute to ^{125}I-PDGF-BB binding, PDGF-AB would be capable of eliminating a greater number of ^{125}I-PDGF-BB-binding sites than PDGF-AA could.

Monoclonal Antibody PR7212 Recognizes the β-Subunit of the PDGF Receptor—We have recently generated a monoclonal antibody, PR7212, against the PDGF receptor (Hart *et al.*, 1987). Two experiments designed to determine whether this antibody recognizes the α- or the β-subunit of the PDGF receptor demonstrated that it recognizes only the β-subunit. In the first experiment, we determined whether PR7212 would immunoprecipitate the different PDGF isoforms when complexed to PDGF receptors. Human fibroblast were incubated with different ^{125}I-labeled PDGF isoforms at 4 °C to occupy PDGF receptors, and then the washed cells were solubilized in detergent to extract ^{125}I-PDGF-receptor complexes. Table II shows that PR7212 immunoprecipitated ^{125}I-PDGF-AB- and ^{125}I-PDGF-BB-receptor complexes but was unable to immunoprecipitate ^{125}I-PDGF-AA-receptor complexes. Thus, PDGF-AB and PDGF-BB, which can bind to receptors con-

TABLE II

Immunoprecipitation of ^{125}I-PDGF-receptor complexes

Immunoprecipitation of ^{125}I-PDGF isoforms bound to the PDGF receptor was performed as described under "Experimental Procedures." The results shown are the amount of ^{125}I-PDGF which was specifically immunoprecipitated as a percentage of the total ^{125}I-PDGF bound to receptors. These data represent the average of triplicate samples. The experiment was repeated with essentially identical results.

^{125}I-PDGF isoform	% of complex specifically immunoprecipitated
AA	1
AB	40
BB	64

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taining β-subunits, were immunoprecipitated, but PDGF-AA, which can only bind to α-subunits, was not. This result suggests that PR7212 recognizes the β-subunit but not the α-subunit.

In the experiment described above, it is possible that ^{125}I-PDGF-AA-receptor complexes were not immunoprecipitated by PR7212 because PDGF-AA but not PDGF-AB or PDGF-BB somehow interfered with antibody recognition of the PDGF receptor rather than because PR7212 does not recognize the α-subunit. We therefore took a second approach to this question by determining which PDGF isoforms would down-regulate binding sites for ^{125}I-PR7212 on MG-63 cells. Fig. 2*b* shows that down-regulation of MG-63 cells with PDGF-BB completely abolished subsequent ^{125}I-PR7212 binding, and down-regulation with PDGF-AB removed greater than 60% of the ^{125}I-PR7212-binding sites. However, PDGF-AA had no effect on ^{125}I-PR7212 binding even though it completely inhibited subsequent ^{125}I-PDGF-AA and ^{125}I-PDGF-AB binding to these cells (Table I). This result confirms that the antireceptor monoclonal antibody PR7212 recognizes only the β-subunit. The subunit model accounts for the quantitative change in ^{125}I-PR7212 binding as follows. PDGF-BB down-regulates all of the β-subunits and totally eliminates ^{125}I-PR7212 binding. PDGF-AB down-regulates all of the α-subunits but only some of the β-subunits and thus partially down-regulates ^{125}I-PR7212 binding. In contrast, PDGF-AA completely down-regulates α-subunits but does not reduce the number of β-subunits and thus fails to reduce ^{125}I-PR7212 binding.

PDGF Induces Association of PDGF Receptor Subunits—An important premise of the receptor subunit model is that PDGF binding requires a stable association of PDGF receptor subunits. To test this prediction of the subunit model, we determined whether PR7212 would immunoprecipitate metabolically labeled mouse receptor subunits via their association with unlabeled human receptor subunits. This approach exploited the fact that PR7212 recognizes human but not mouse PDGF receptors (Hart *et al.*, 1987). 3T3 cell receptors were metabolically labeled with [^{35}S]methicnine followed by extraction with detergent. These extracts were incubated with detergent extracts of unlabeled human fibroblasts in the presence or absence of the different individual PDGF isoforms, and then PR7212 or a control monoclonal IgG was added to immunoprecipitate the human β-subunits. The precipitates were then analyzed by SDS-PAGE under reducing conditions. As shown in Fig. 4*a*, PR7212 specifically immunoprecipitated the 180-kDa mouse PDGF receptor subunits only if PDGF-AB or PDGF-BB had been added to the mixed extracts to form human-mouse receptor complexes. In the absence of PDGF or if only PDGF-AA was present, PR7212 did not precipitate mouse receptor subunit. Since PR7212 does not directly recognize mouse PDGF receptor subunits, this result indicates that PDGF receptor subunits enter into stable associations only when exposed to PDGF. Most importantly, the result conforms to the predicted specificity of the PDGF receptor subunits for the binding of the different PDGF isoforms. Thus PDGF-AA, which does not bind to β-subunits, could not cause association of mouse subunits with human β-subunits (Fig. 4*a*). PDGF-AB and PDGF-BB can incorporate both α- and β-subunits and thus resulted in association of human to mouse PDGF receptor subunits and thereby allowed immunoprecipitation by PR7212.

In the previous experiment, the subunit model predicts that PDGF-AB caused precipitation of only mouse α-subunits since the B-chain of PDGF-AB must be bound to a human β-subunit (in order to be recognized by PR7212), and the A-chain can bind only α-subunits (see Fig. 3*c*). To test the pattern of subunit association further, we repeated the above experiment with the following modification. After metabolically labeling the 3T3 cells, one set was down-regulated with PDGF-AA to eliminate labeled mouse α-subunits. Consistent with the subunit model, addition of PDGF-AB results in immunoprecipitation of mouse receptor subunits from control extracts but not from extracts made from the PDGF-AA down-regulated cells (Fig. 4*b*). By comparison, elimination of labeled mouse α-subunits by PDGF-AA down-regulation results in only a partial reduction of the number of mouse subunits immunoprecipitated by PR7212 following incubation with PDGF-BB, consistent with the ability of PDGF-BB to incorporate either mouse α- or β-subunits (see Fig. 3*c*). These experiments demonstrate the association of mouse and human PDGF receptors in the PDGF isoform-specific pattern predicted by the subunit model.

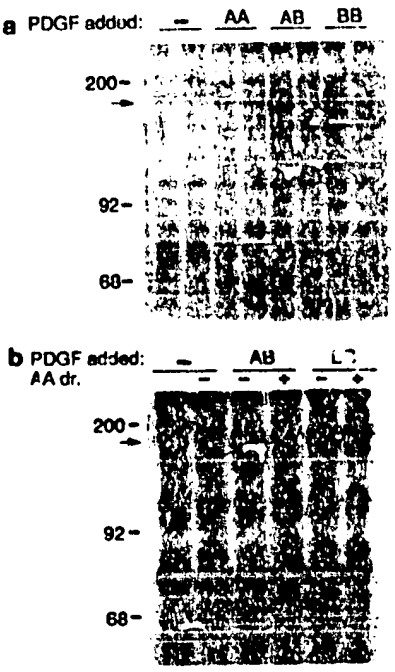

FIG. 4. **Coimmunoprecipitation of metabolically labeled 3T3 receptors with human receptors using monoclonal PR7212.** *a*, extracts of Swiss/3T3 cells metabolically labeled overnight with [^{35}S]methionine were mixed with extracts of unlabeled human fibroblasts and incubated with the three PDGF isoforms as indicated. Human PDGF receptors were immunoprecipitated using PR7212 (*left lanes*) or a control monoclonal antibody (*right lanes*) and the proteins analyzed by SDS-PAGE under reducing conditions followed by autoradiography. *b*, the above experiment was repeated except that some of the metabolically labeled 3T3 cells were down-regulated with PDGF-AA for 2 h prior to detergent extraction (*AA dr*). The *first lane* was immunoprecipitated with a control monoclonal antibody and all *other lanes* with PR7212.

The Number and Proportions of Subunits on Different Cell Types—The total number of PDGF receptors was determined by saturation binding using ^{125}I-PDGF-BB (Fig. 1, AG1523 data not shown) since this isotype binds to all classes of PDGF receptors. Since PDGF-AA binds only to α-subunits, the relative number of α- *versus* β-subunits was determined from the fraction of ^{125}I-PDGF-BB binding which was competed for by unlabeled PDGF-AA (Table I). Fig. 5 summarizes the results of this analysis on the cell types examined in this study. Human fibroblasts express 10-fold more PDGF receptors than do U-2 OS cells, yet both cell types express the

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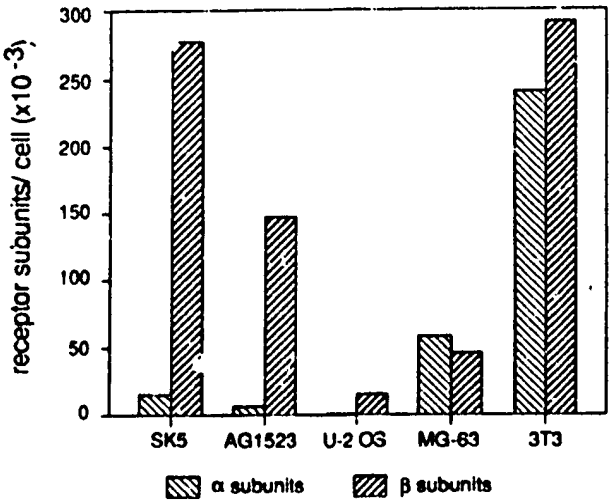

FIG. 5. **Calculated number of PDGF receptor α- and β-subunits on human cells.** The total number of PDGF receptors was determined from the number of ^{125}I-PDGF-BB-binding sites as measured in Fig. 1 (data for AG1523 not shown in Fig. 1). The percentage of α- *versus* β-subunits was calculated from the fraction of ^{125}I-PDGF-BB binding which could be competed for by PDGF-AA (Table I). The number of receptor subunits was calculated (assuming that high affinity receptors are dimers of subunits) as twice the number of ligand-binding sites.

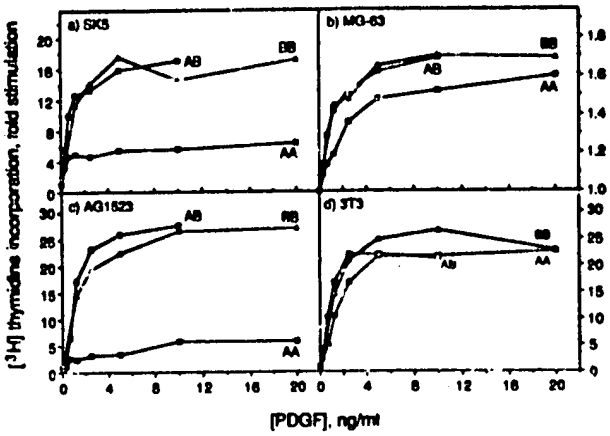

FIG. 6. **Mitogenic effect of PDGF isoforms.** Stimulation of [^{3}H]thymidine incorporation in response to the different PDGF isoforms was determined as described under "Experimental Procedures." The results are plotted as fold stimulation of [^{3}H]thymidine incorporation over basal levels and represent the mean of triplicate wells.

same ratio of the two receptor subunits, *i.e.* almost 20-fold more β- than α-subunits. In contrast, MG-63 osteosarcoma cells have more α-subunits than any of the other human cell lines examined, yet unlike any of the other human cells, they have a nearly equal number of α- and β-subunits. The Swiss/3T3 cells also have approximately equal numbers of α- and β-subunits, but they express 4–5-fold more subunits than do MG-63 cells. Thus, they have approximately the same number of β-subunits and 10-fold more α-subunits than do human fibroblasts.

The Mitogenic Efficacy of PDGF Isoforms Varies on Different Cells—PDGF-AA has been reported to be only weakly mitogenic for human fibroblasts (Nister *et al.*, 1988; Heldin *et al.*, 1988). Since fibroblasts express relatively few α-subunits, it seemed possible that the poor mitogenic response reflected a low number of PDGF-AA receptors rather than an intrinsically low mitogenic potency of PDGF-AA. In agreement with these previous reports, two strains of human fibroblasts, SK5 and AG1523, responded well to PDGF-AB and PDGF-BB, whereas PDGF-AA stimulated a much lower level of maximal [^{3}H]thymidine incorporation (Fig. 6). Although PDGF-AA was less efficacious than the other two PDGF isoforms, it was equally potent, *i.e.* half-maximal stimulation of [^{3}H]thymidine incorporation occurred at concentrations of 0.5–2.5 ng/ml for all three PDGF isoforms. In contrast, all three isoforms were equally effective in stimulating [^{3}H]thymidine incorporation in 3T3 and MG-63 cells (Fig. 6) which express significantly more α-subunits than do fibroblasts. The maximalfold stimulation of [^{3}H]thymidine incorporation in MG-63 cells was quite small compared with any of the other cells tested due to the relative inability to bring this transformed cell population to a growth-arrested state; nevertheless, within the range that regulation was possible, all three PDGF isoforms were equally effective.

DISCUSSION

The Receptor Subunit Model—We propose a new model for the structure of the PDGF receptor based on an analysis of the unusual pattern of binding competition of the three PDGF isoforms. A key element of the subunit model is that high affinity binding of PDGF requires formation of at least a dimeric receptor from subunits within the plasma membrane. Formation of dimeric or multimeric receptors was demonstrated by the ability to coimmunoprecipitate mouse and human receptor subunits in a PDGF isoform-specific manner (Fig. 4).

The subunit composition of PDGF receptors appears to be determined in response to binding to the different PDGF isoforms. PDGF-AA binds only to αα-receptors yet competes almost completely for binding of ^{125}I-PDGF-AB (Table I) which could also bind to αβ-receptors. The model accounts for this since the initial exposure to PDGF-AA would drive all of the α-subunits into αα-receptors, and none would be left to form αβ-receptors. Conversely, receptor α-subunits can be driven into αβ-receptors if the cells are exposed to PDGF-AB. This recruitment of β-subunits is necessary to explain how PDGF-AB can compete for more ^{125}I-PDGF-BB binding than does PDGF-AA (Fig. 2 and Table I) which can bind only to α-subunits. The ability of the different PDGF isoforms to determine receptor subunit association suggests that unoccupied PDGF receptor subunits exist either as monomers or as dimers whose subunits are in rapid equilibrium.

We have recently obtained additional evidence for the existence of distinctly different components of the PDGF receptor. We have generated a monoclonal antibody that is specific for the α-subunit of the PDGF receptor, and its use in metabolic pulse-chase experiments with human cells indicates that the two receptor subunits are slightly different in size as determined by SDS-PAGE.[2] Although the proteins immunoprecipitated by PR7212 and this new monoclonal antibody are distinguishably different in size when run on different lanes, they are very difficult to resolve when combined in a single lane and thus are not resolved in Fig. 4. We have also recently cloned a partial human cDNA that has a nucleotide sequence very similar to but distinctly different from the recently reported cDNAs for the PDGF receptor.[3] The previously reported (Gronwald *et al.*, 1988) and newly cloned cDNAs specifically hybridize to different sized transcripts (5.7 *versus* approximately 6.0 kilobases). A survey of

[2] E. M. Blackwood, R. S. Serfert, and D. F. Bowen-Pope, manuscript in preparation.

[3] J. D. Kelly and D. F. Bowen-Pope, unpublished observations.

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different cell types for transcripts recognized by this newly acquired partial cDNA is consistent with the gene product encoding for the α-subunit of the PDGF receptor.

It has been proposed that receptor dimerization may be required for high affinity binding of ligand in an increasing number of growth factor receptor systems. ^{125}I-Insulin binds to insulin $\alpha\beta$-receptors (functionally monomeric) with lower affinity than to $\alpha_2\beta_2$-receptors (functionally dimeric) (Boni-Schnetzler *et al.*, 1987; Sweet *et al.*, 1987). The interleukin-2 receptor is also composed of α- and β-subunits. Interleukin-2 binds to each of these two subunits with low affinity, while it binds to the noncovalently associated $\alpha\beta$-receptor complex with 100–1000-fold higher affinity (Smith, 1988). Unoccupied epidermal growth factor receptors are thought to exist on the cell surface in both a monomeric and dimeric state. Epidermal growth factor binds to dimeric receptors with higher affinity than to monomeric receptors, epidermal growth factor binding has been proposed to shift the monomeric-dimeric receptor equilibrium to the dimeric form (Yarden and Schlessinger, 1987; Boni-Schnetzler and Pilch, 1987). A model with strong similarity to the proposed model for the PDGF receptor system has been suggested in which high affinity binding of nerve growth factor occurs to dimeric nerve growth factor receptors cross-linked by the dimeric ligand (Buxser *et al.*, 1985).

The proposed model for the structure of the PDGF receptor differs from these other systems in at least one important aspect. Unlike the ligand-receptor systems described above, PDGF is composed of two distinct proteins that can be combined to create three different isoforms. We are proposing that formation of dimeric receptors not only results in *high affinity* binding but that the receptor subunit composition determines PDGF isoform binding *specificity*. The PDGF isoform binding specificity to the different receptor classes can be nearly absolute. For example, PDGF-AA competition for ^{125}I-PDGF-BB binding to MG-63 cells reaches a level plateau as determined by the relative number of the two receptor subunits (Fig. 2). Since higher concentrations of this ligand did not result in additional competition, there is no evidence for low affinity interaction with PDGF receptor β-subunits. However, it does appear that PDGF-AB may bind to β-subunits with low affinity (see below).

Relationship of the Receptor Subunit Model to Previous Models—We recently reported (Hart *et al.*, 1988) that there are two classes of monomeric PDGF receptors as opposed to the proposal here that there are two subunits which can form three types of high affinity receptor. Heldin *et al.* (1988) have also recently published observations that suggested only two classes of PDGF receptor on human fibroblasts. Their data are in good agreement with our original model and are consistent with our current hypothesis except in one detail. They observed that PDGF-AB competed for a significant fraction of ^{125}I-PDGF-BB binding on human fibroblasts. Given the relative number of the two receptor subunits on human fibroblasts, such competition is not expected, and we did not observe it in our studies. However, if the purified platelet PDGF-AB used in their binding competition experiments contained just a small amount of PDGF-BB, then all of their observations would coincide exactly with ours. Hammacher *et al.* (1988) have subsequently shown that the PDGF-AB used by Heldin *et al.* (1988) does contain PDGF-BB (30% by mass).

Additional evidence consistent with the existence of multiple classes of PDGF receptors has come from recent reports on the PDGF-binding properties of cells transfected with cDNAs for the human PDGF receptor. Baby hamster kidney and Chinese hamster ovary cells transfected with a cDNA for the human receptor bind ^{125}I-PDGF-BB with high affinity, ^{125}I-PDGF-AB with low affinity, and ^{125}I-PDGF-AA not at all (Claesson-Welsh *et al.*, 1988; Gronwald *et al.*, 1988). These results are consistent with the cloned cDNAs encoding for the human PDGF receptor β-subunit which form $\beta\beta$-receptors that will only bind PDGF-BB with high affinity and will not bind PDGF-AA. PDGF-AB binds with low affinity to either $\beta\beta$-receptors or monomeric β-subunits.

Another recently published report concluded that Chinese hamster ovary cells transfected with a human PDGF receptor cDNA express a single class of receptor which binds both PDGF-AA and PDGF-BB (Escobedo *et al.*, 1988). Although the actual cDNA sequence was not reported, other evidence suggests that this is a cDNA for the same gene which, when expressed in baby hamster kidney or Chinese hamster ovary cells, gave high affinity binding of ^{125}I-PDGF-BB (Claesson-Welsh *et al.*, 1988; Gronwald *et al.*, 1988) but did not bind PDGF-AA. Two possibilities may explain this discrepancy. First, Escobedo *et al.* (1988) may have cloned a cDNA for the α-subunit; our model would predict that the product of this gene would have the binding properties they observed. Alternatively, it is possible that the cloned transfectants they selected are expressing endogenous α-subunits. We and others have observed that at least some populations of Chinese hamster ovary cells express low levels of endogenous PDGF receptors (Bowen-Pope *et al.*, 1985; Claesson-Welsh *et al.*, 1988).

An unresolved question of considerable interest is the relationship between the two receptor subunits. Heldin *et al.* (1988), using an affinity cross-linking approach, have reported that PDGF-AB and PDGF-BB bind to two size classes of PDGF receptor and that PDGF-AA binds only to the smaller class. This result would suggest that the PDGF receptor α-subunit is smaller than the β-subunit. In performing similar affinity cross-linking studies, we also observed PDGF isoform-specific binding to multiple size classes of PDGF receptor.[3] Other data, however, suggest that the subunits are very similar in size. In the coimmunoprecipitation experiment of Fig. 4, addition of PDGF-AB should result in precipitation only of labeled α-subunits, while addition of PDGF-BB should have coprecipitated both labeled α- and β-subunits, yet only a single size band is observed in both of these cases. Similarly, two-dimensional gel analysis of PDGF receptors phosphorylated on tyrosine in response to the different PDGF isoforms suggests that the α- and β-subunits are indistinguishable in their size and charge properties (Kazlauskas *et al.*, 1988). The differences in size apparently detected in the cross-linking experiments might owe more to differences in the way the labeled ligands were cross-linked to the receptor than to differences in the receptor *per se*. Nevertheless, the two subunits must possess physical differences to account for their different binding specificities and the ability of the antireceptor monoclonal antibody PR7212 to recognize the β-subunit but not the α-subunit. Are the two receptor subunits encoded for by separate genes, or are they derived from the same gene with the different binding properties reflecting differences in post-translational processing or association with "accessory proteins?" The cDNA transfection experiments discussed above are most consistent with the two subunits being products of separate genes. Stronger evidence that the α-subunit is encoded by a separate gene is the observation that MG-63 cells tranfected with an antisense construction of the human PDGF receptor cDNA show complete loss of expression of the β-subunit with no change in levels of α-subunit.[3] Finally, as noted above, we have cloned a partial cDNA that appears

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to encode for the α-subunit, which would suggest that the two subunits are encoded for by separate genes.

Biological Effects—We have shown that the relative number of the two types of PDGF receptor subunits varies significantly between cell types (Fig. 5). This suggested the possibility that the relative mitogenic potency of the different PDGF isoforms for different cells might be dictated in part by the level of expression of the relevant subunit rather than by differences in the intrinsic potencies of the isoforms. We found that PDGF-AA is a less effective mitogen for human fibroblasts (which express few α-subunits) than is PDGF-AB or PDGF-BB (Fig. 6). A similar result was obtained by Nister *et al.* (1988) who reported that a PDGF-AA-like growth factor purified from glioma cells was poorly mitogenic for human fetal fibroblasts. However, the PDGF-AA isoform is not intrinsically a poor mitogen since it elicited as much stimulation of DNA synthesis in 3T3 and MG-63 cells (both of which express higher levels of α-subunits) as did PDGF-AB and PDGF-BB (Fig. 6 and Escobedo *et al.*, 1988).

We have recently found large differences in the total amounts and in the relative proportions of the different PDGF isoforms in serum from different species and in medium conditioned by cultured cells (Bowen-Pope *et al.*, 1989). The PDGF in animal sera (except for human) and in medium conditioned by cultured endothelial cells is largely the PDGF-BB isoform. In contrast, vascular smooth muscle cells from neonatal rats and a variety of cultured transformed cells secrete almost no detectable PDGF-BB into their medium. Therefore, sources of PDGF vary in the composition of PDGF isoforms, and PDGF-responsive cells differ in expression of the two receptor subunits. This variability may allow for a finely tuned regulation of cellular responsiveness to PDGF under different physiological conditions.

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Note Added in Proof—Since submission of this manuscript, two papers have been published which confirm the existence of two distinct PDGF receptor proteins. Claesson-Welch *et al.* (Claesson-Welch, L., Hammacher, A., Westermark, B., Heldin, C.-H., and Nister, M. (1989) *J. Biol. Chem.* **264**, 1742–1747) have demonstrated two PDGF receptor proteins that differ in size and antigenicity, and Matsui *et al.* (Matsui, T., Heidaran, M., Miki, T., Popescu, N., Le Rochelle, W., Kraus, M., Pierce, J., and Aaronson, S. (1989) *Science* 243, 800–804) have obtained a cDNA for a second distinct PDGF receptor protein.

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